PUBLISHABLE SUMMARY

Dendritic cells (DCs) are divided in multiple subsets, each one with distinct functions at the interface of the innate and adaptive immunity. Two major subsets of DCs circulate in human peripheral blood: the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs), which can be distinguished by the expression of specific surface markers. Within mDCs, slanDCs are the most abundant in human blood and are characterized by a specific phenotype (6-sulfo LacNAc^{+}, CD11c^{+}, CD1c^{-}, CD14$^{low/-}$, CD16^{+}, CD45RA^{+}, C5aR^{+}). Since their identification in 1998, subsequent studies have revealed slanDCs as cells endowed with strong pro-inflammatory properties. slanDCs display a potent ability to secrete large amounts of pro-inflammatory cytokines such as TNFα and interleukin-12p70 (IL-12p70) upon stimulation with bacterial lipopolysaccharide (LPS) or toll-like receptor 8 (TLR8) agonists and the capacity to promote Th1 and Th17 cell polarization. In addition, many studies have revealed the central role of slanDCs in the pathophysiology of numerous diseases including psoriasis, Crohn's disease, rheumatoid arthritis, chronic forms of allergy and lupus erythematosus. Furthermore, evidence of a novel network between natural killer (NK) cells, polymorphonuclear neutrophils (PMN) and slanDCs confirm the relevant contribution of slanDCs in the immune system response. The central importance of slanDCs in immune regulation makes, therefore, additional studies necessary to better understand the biologic role of this DC subset. For such a purpose, this proposal has been focused on three main objectives:

1. Studies of the molecular mechanisms regulating IL-12p70 production by slanDCs during their maturation.

IL-12p70 is a heterodimeric cytokine, formed by IL-12p35 and IL-12p40, that plays a key role in the initiation of innate and adaptive immune responses favoring Th1 polarization and IFN-γ production. slanDCs undergo spontaneous maturation if cultured in the absence of erythrocytes and once matured, they are characterized by a high potential to produce IL-12p70 in response to LPS.

Thus, slanDC phenotypical maturation during in vitro culture was confirmed by an increased expression of CD83, HLA-DR, CD86 and CD40 cell surface markers. slanDC maturation process was also characterized by an augmented expression of TLR4 and its co-receptor CD14, indicating that the presence of a higher number of receptor molecules might be implicated in the increased capacity by mature slanDCs to produce IL-12p70 in response to LPS compared to immature ones. However, this mechanism was ruled out since spontaneously matured slanDCs expressed higher levels of IL-12p35 and IL-12p40 mRNA upon LPS-treatment than freshly isolated slanDCs, but not of other cytokines such as TNFα. Therefore, we concluded that the molecular bases regulating the production of IL-12p35 and IL-12p40 chains prior to, and after, slanDC maturation are gene-specific. Furthermore, we observed that the effect of slanDC maturation on IL-12p35 and IL-12p40 mRNA levels upon LPS stimulation is also cell-specific since, in contrast to slanDCs, no differences in IL-12p35 and IL-12p40 mRNA expression between immature and mature LPS-stimulated CD14^{+} monocytes isolated from the same donor were found. The next objective was to study the putative epigenetic mechanisms involved in the regulation of IL-12 gene expression in slanDCs. Covalent modifications of histones, such as methylation and acetylation of specific residues, are involved in regulating chromatin organization and, in turn, in the control of gene expression. Chromatin immunoprecipitation (ChIP) assays using specific antibodies for modified histones allow the detection of histone posttranslational modifications and the establishment of chromatin marks associated with different states of gene activity. Therefore, to understand whether an alteration of epigenetic regulatory mechanisms at the level of IL-12p35 and IL-12p40 loci explains the greater capacity to produce IL-12p70 by mature slanDCs, we performed ChIP assays using antibodies towards tetraacetylated histone H4 (H4Ac), which is enriched in active euchromatin, and histone H3 monomethylated at K4 (H3K4me1), which is detected at both promoters and enhancers. Interestingly, preliminary data would show that the IL-12p40 genomic locus might be poised for activation after slanDC maturation suggesting an epigenetic mechanism regulating IL-12p70 production by slanDCs.

2. Investigation on the molecular mechanisms whereby LPS regulates slanDC survival, in the absence or the presence of neutrophils.

Apoptotic cell death is a fundamental process to regulate the equilibrium between tolerance and an effective immune response. Apoptosis can proceed through two different signaling cascades denominated extrinsic pathway, which is death receptor-dependent, and intrinsic pathway, which is mitochondria mediated.

The initial approach to this task was to establish whether the killing effect induced by LPS on slanDCs was mediated by extracellular signals through autocrine/paracrine mechanisms and/or modulation of death receptor expression. Thus, we have concluded that the binding of lethal ligands, such as FasL/CD95L, TNFα and TRAIL, to death receptors (Fas/CD95, TNFα receptor 1 (TNFR1) and TRAIL receptor (TRAILR)1–2, respectively) is not implicated in LPS-induced apoptosis in slanDCs. This finding was in agreement with an increase of caspase 9 and 3 activities but not of caspase 8,

the latter one which is activated in death receptor-mediated extrinsic pathways only. Moreover, the addition of LPS to the slanDC culture transcriptionally upregulated the c-FLIP gene, which encodes an anti-apoptotic regulator protein which inhibits death receptor-mediated cell death. After excluding the possible involvement of death receptor activation as responsible of LPS-induced slanDC apoptosis, the role of reactive oxygen species (ROS), well-known intrinsic apoptosis inductors, was investigated in such an effect. As such, we have demonstrated, by a number of assays, that LPS-stimulated slanDCs produce ROS and that they are susceptible to oxidant-induced cell death upon cell incubation with H_2O_2 at concentrations comparable to the amounts of superoxide anion produced by LPS-stimulated slanDCs. Furthermore, addition of N-acetyl-cysteine, an antioxidant peptide, partially reduced the apoptotic cell percentage in LPS-treated slanDCs. Finally, catalase and gluthathione peroxidase, both enzymes implicated in H_2O_2 clearance, genes were downregulated upon LPS stimulation. Thus, all these data indicate that an oxidative process induced upon LPS stimulation may be involved in its pro-apoptotic effect on slanDCs.

In addition, in order to study the mechanisms regulating the survival of slanDCs upon stimulation with LPS in the presence of neutrophils, we performed co-culture assays. Results derived from these experiments indicated that co-culture of slanDCs with neutrophils results in a reduction of LPS-induced slanDC apoptosis, and that this neutrophil-mediated slanDC survival occurs in a cell-contact dependent but ICAM-1 and CD18-independent manner.

3. Studies on the pattern of chemokine/chemokine receptor expression by slanDCs and their migration capacity.

slanDCs have been found in inflamed tissues of Crohn's disease and psoriasis, co-localized with other immune cells such as neutrophils and NK cells. Thus, a better understanding of the molecular and cellular events of the migration ability of slanDCs and their capability of directing the recruitment of other cell types is crucial for extending our knowledge on the pathogenesis of diseases.

A cytofluorimetric analysis of a panel of 12 different chemokine receptors showed that only CX3CR1 and CXCR4 are expressed in slanDCs at high and low levels, respectively. Moreover, complement component C5a receptor (C5aR) is also present on slanDC surface. Gene microarray studies performed in isolated slanDCs confirmed high mRNA expression levels of CX3CR1, CXCR4 and C5aR. Moreover, slanDC chemotaxis assays showed that either fractalkine (CX3CL1), the ligand of CX3CR1, or complement component C5a induce a potent slanDC migration, whereas stromal cell-derived factor 1 (SDF-1), the CXCR4 ligand which is also known as CXCL12, produce a discrete slanDC chemotaxis. However, preliminary experiment performed in order to study the possible induction of slanDC migration by other immune cell types, showed that supernatants from activated neutrophils do not induce chemotaxis of slanDCs. In addition, we investigated the effect of active slanDCs on the recruitment of neutrophils, which express CXCR1 and CXCR2 on their surface. Either nonstimulated slanDC supernatants or, more strongly, LPS-treated ones induced neutrophil migration. Moreover, this chemotaxis was completely, in the case of nonstimulated slanDC supernatant, and partially, in the case of LPS-treated slanDC supernatants, suppressed by an anti-CXCR1/2 antibody. CXCL8, a known ligand of CXCR1 and CXCR2, was detected in slanDC supernatants in basal conditions and up to 10 times higher concentration upon LPS stimulation. Thus, CXCL8 might be considered the main candidate for neutrophil chemotaxis induced by slanDCs, however we should also consider a putative role of CXCL1, CXCL2 and CXCL3, ligands of CXCR2, whose mRNA levels, established by microarray studies, were shown to be increased upon LPS stimulation of slanDCs.

Finally, analysis of cell transcriptome (i.e., expression of thousands of genes) has been performed by microarray studies in slanDCs incubated with or without LPS. Bioinformatic analysis of these data has provided a complementary information for the previously described tasks and for the knowledge of slanDC biology.

Undoubtedly, results obtained from this project has provided new knowledge on the functional specialization of human slanDCs, and, therefore, new concepts to immune disease pathogenesis and design of novel therapies.

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